Upregulation of miR-6747-3p affects red blood cell lineage development and induces fetal hemoglobin expression by targeting BCL11A in β-thalassemia

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Abstract. In β-thalassemia, excessive α-globin chain impedes the normal development of red blood cells resulting in anemia. Numerous miRNAs, including miR-6747-3p, are aberrantly expressed in β-thalassemia major (β-TM), but there are no reports on the mechanism of miR-6747-3p in regulating red blood cell lineage development and fetal hemoglobin (HbF) expression. In the present study, RT-qPCR was utilized to confirm miR-6747-3p expression in patients with β-TM and the healthy controls. Electrotransfection was employed to introduce the miR-6747-3p mimic and inhibitor in both HUDEP-2 and K562 cells, and red blood cell lineage development was evaluated by CCK-8 assay, flow cytometry, Wright-Giemsa staining and Benzidine blue staining. B-cell lymphoma/leukemia 11A (BCL11A) was selected as a candidate target gene of miR-6747-3p for further validation through FISH assay, dual luciferase assay and Western blotting. The results indicated that miR-6747-3p expression was notably higher in patients with β-TM compared with healthy controls and was positively related to HbF levels. Functionally, miR-6747-3p overexpression resulted in the hindrance of cell proliferation, promotion of cell apoptosis, facilitation of cellular erythroid differentiation and γ-globin expression in HUDEP-2 and K562 cells. Mechanistically, miR-6747-3p could specifically bind to the 546-552 loci of BCL11A 3'-UTR and induce γ-globin expression. These data indicate that upregulation of miR-6747-3p affects red blood cell lineage development and induces HbF expression by targeting BCL11A in β-thalassemia, highlighting miR-6747-3p as a potential molecular target for β-thalassemia therapy.

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Introduction

β-thalassemia is a widespread recessive hereditary disease characterized by inadequate or ineffective composition of β-globin, anemia and ineffective erythropoiesis. It has been reported that 80-90 million individuals have β-thalassemia worldwide (1). Due to severe hypoxia caused by anemia, patients with β-thalassemia major (β-TM) often have hepatosplenomegaly, growth retardation, jaundice, pale complexion and marrow expansion (2). Current treatment strategies for β-thalassemia, including thalidomide, deferasirox, deferiprone, iron chelation, splenectomy, blood transfusion and hematopoietic stem cell transplantation still have numerous drawbacks including difficulty in donor matching and graft rejection in addition to a high cost (3,4). Novel treatment methods, including gene therapy and gene editing, have been previously investigated, and relevant clinical trials have shown improvements in anemia in patients with β-thalassemia (5-8). Furthermore, it has been demonstrated that the novel activin receptor ligand trap Luspatercept improves late-stage erythropoiesis (9,10). Despite the promising nature of these methods, there remain numerous safety concerns including off-target activity and chromosomal rearrangement events due to the small number of clinical trials conducted (11,12). Therefore, it is crucial to verify novel approaches for managing β-thalassemia.

Human hemoglobin (Hb) undergoes two switches from the embryonic to the postnatal period, in which the main Hb changes from fetal hemoglobin (HbF; $\alpha_2\gamma_2$) to adult hemoglobin (HbA; $\alpha_2\beta_2$) (13). This process is affected by several transcription factors, including B-cell lymphoma/leukemia 11A (BCL11A), activating transcription factor 4, Kruppel-like factor 1, v-myb avian myeloblastosis viral oncogene homolog, specificity protein 1 and Ly1 antibody reactive (14-16).

However, the upstream regulators of BCL11A remain incompletely characterized (4). Currently, a promising way to treat β-thalassemia is γ-globin reactivation, which elicits the point mutation of hereditary persistence of HbF in patients (17).

miRNAs are groups of non-coding RNA molecules and are essential for various biological functions such as cell differentiation, maturation and proliferation (18,19). Certain microRNAs, such as miR-32, influence early erythroid commitment; miR-22 and miR-28 have been shown to impact the maturation of erythroid cultures *in vitro* maturation (20-22). Normal erythropoiesis is characterized by a significant rise in miR-155, while the expression pattern of miR-339 is biphasic (23). By changing the lifespan of globin chains, these miRNAs control the production of Hb, iron metabolism and resistance to oxidative stress in red blood cells (24).

miRNA sequence tests were conducted as in a previous study on the peripheral blood of patients with β-TM and the healthy controls to detect miRNAs with varying expression levels (23). Out of the differently expressed 196 miRNAs, miR-6747-3p was identified as being notably increased (fold change, 4.76; P=0.001) and showing a positive association with HbF (25). Current studies about miR-6747-3p have focused on the direction of endometriosis (26), Alzheimer's disease (27) and small cell lung cancer (SCLC) (28). However, whether miR-6747-3p plays a role in hematologic diseases remains unclear. By conducting *in vitro* functional experiments, the present study aims to identify the expression of miR-6747-3p in patients with β-TM, in addition to seeking its regulatory impacts on red blood cell lineage development of erythroid precursor cells and γ-globin expression.

Materials and methods

Patient enrollment. The Ethics Committee of the Fujian Maternity and Child Health Hospital authorized the present study (Fuzhou, China; approval no. 2019073), which followed the Helsinki Declaration. Peripheral blood samples were collected from 20 patients with β-TM (age, 8.30±1.59 years; female/male, 13/7) and 20 healthy controls (age, 9.00±2.23 years; female/male, 12/8) before blood transfusion or hydroxyurea treatment at Fujian Maternity and Child Health Hospital (Fuzhou, China) between January 2020 to December 2021. For the group comprised of patients with β-thalassemia, the inclusion criteria included patients exhibiting anemia symptoms (Hb <90 g/l; normal reference value in children aged 6 months to 6 years, 105-140 g/l; normal reference value in children aged 7-12 years, 110-160 g/l) and carrying β^0/β^0 (n=16), β^0/β^+ (n=2), β^+/β^+ (n=2) genotypes. Control groups were those age-matched individuals with normal thalassemia gene diagnosis and peripheral blood indexes. The exclusion criteria included: i) Patients with asthma, epilepsy and diabetes; ii) patients with acute and chronic lung infection; iii) patients with abnormal blood coagulation; and iv) patients with α-thalassemia, iron deficiency anemia and megaloblastic anemia. All patients or their guardians provided written informed consent.

Sample collection. Peripheral blood samples were gathered as previously described (29). Briefly, 5 ml peripheral blood from the participants was preserved and isolated using a PAX gene blood RNA kit (Qiagen GmbH). Analysis was conducted using the Sysmex XN-3000 automated hematology analyzer (Sysmex Corporation) to evaluate blood cell parameters. An automated capillary electrophoresis device (version 6.2; Sebia) was used to analyze the Hb composition and levels.

Cell culture and transfection. The human umbilical cord blood-derived erythroid progenitor (HUDEP-2) cells were provided by RIKEN BioResource Centre through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology (Tsukuba, Ibaraki, Japan). The cells were cultured in a serum-free StemSpan SFEM® medium (Stemcell Technologies, Inc.), supplemented with 3 IU/ml erythropoietin (EPO; Amgen, Inc.), 1 μg/ml doxycycline (Sigma-Aldrich; Merck KGaA) and $1x10^{-6}$ M dexamethasone (Sigma-Aldrich; Merck KGaA). K562 cells derived from human erythroleukemia were acquired from Shanghai Anwei Biotechnology Co., Ltd. All cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

The miR-6747-3p mimic (5'-UCCUGCCUUCCU CUGCACCAG-3') and its negative control (5'-UUCUCC GAACGUGUCACGUTT-3'), along with the miR-6747-3p inhibitor (5'-CUGGUGCAGAGGAAGGCAGGA-3') and its control inhibitor (5'-CAGUACUUUUGUGUAGUA CAA-3') were obtained from Shanghai Genepharma Co., Ltd. HUDEP-2/K562 cell transfections were carried out by Amaxa Nucleofector II Device (Lonza Group, Inc.) according to the manufacturer's instructions at room temperature. The reagents used for electroporation of HUDEP-2 and K562 cells were Cell Line Nucleofector™ Kit (Lonza Group, Inc.), and the electroporation programs were U-008 and ATCC. After electroporation with oligonucleotides at 100 nM concentration at room temperature for 2 sec, the cells were cultured in a 37˚C incubator for 48 h for flow cytometry, cell cycle detection and cell RNA extraction. The cell protein was extracted after 72 h. K562 cells were induced by Hemin for 96 h for cell differentiation detection and benzidine staining. HUDEP-2 cells were cultured in three-stage medium for 14 days before erythroid differentiation testing and Wright-Giemsa staining.

RNA extraction and qPCR analysis. Total RNA of peripheral blood samples and HUDEP-2/K562 cells were gathered by the PAX Gene Blood RNA Kit (Qiagen GmbH) and the Eastep® Super Total RNA Extraction Kit (Promega Corporation) following the manufacturer's guidelines. The RNA was quantified by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The cDNA of miRNA and mRNA were generated with Mir-X™ miRNA First-Strand Synthesis Kit and PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio, Inc.). γ-globin, BCL11A and miR-6747-3p relative expression levels were computed by applying the comparative cycle threshold approach. The StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was adopted to perform miRNA and mRNA qRT-PCR with TB Green® Advantage® qPCR Premix and TB Green® Premix Ex Taq™ II Kit (Takara Bio, Inc.,), according to the manufacturer's instruction. The miRNA RT-qPCR protocols were demonstrated as follows: After a 10 sec denaturation stage, there were 40 cycles of incubation at 95˚C for 5 sec, 62˚C for 20 sec and 55˚C for 30 sec. The RT-qPCR detection

protocol for mRNA was conducted as follows: After a 30 sec pre-denaturation phase, a total of 40 amplification cycles were executed, comprising denaturation at 95˚C for 5 sec, annealing at 60˚C for 34 sec, and extension at 95˚C for 15 sec. The $2^{-\Delta\Delta Cq}$ technique was utilized to determine the relative fold-change of each target gene in relation to GAPDH and U6 (30). The primer sequences are available in Table SI.

Cell proliferation assessment. The Cell Counting Kit-8 (CCK8; APeXBIO Technology LLC) was utilized to quantify cell proliferation ratio. HUDEP-2 and K562 were seeded in 96-well plates after adding 10 μl CCK-8 reagent to each well. After 2 h of incubation at 37˚C, the cell viability was assessed using a microtiter reader (Thermo Fisher Scientific, Inc.) at 450 nm. Proliferation of the cells was assessed at 0, 24, 48, 72 and 96 h. Every experiment was run three times.

Flow cytometry assay. The impacts of miR-6747-3p on cell cycle and apoptosis were examined using flow cytometry. A total of 5 μl propidium iodide (PI; BD Biosciences) were added to stain HUDEP-2 and K562 cells after fixation with 75% ethanol at -20˚C overnight. The G0/G1, S and G2/M ratios were analyzed using ModFit software (V3.2.; Verity Software House, Inc.). Annexin V-FITC/PI (BD Biosciences) was used to stain cells for 30 min at room temperature following the manufacturer's guidelines to detect cell apoptosis. After which, BD LSRFortessa™ X-20 (BD Biosciences) and FlowJo software V10 (FlowJo LLC) were used to assess the apoptosis experiments.

Erythroid differentiation test. A three-phase differentiation protocol was used to differentiate HUDEP-2 cells (31). The procedure involved three phases including phase 1 (days 1-4), using Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 100 ng/ml Stem Cell Factor (SCF), 10 μg/ml recombinant human insulin, 5% human AB serum, 1% L-glutamine, 330 μg/ml holo-transferrin, 3 U/ml EPO, 1 μg/ml doxycycline, 2 U/ml heparin and 1% penicillin/streptomycin. Phase 2 (days 5-7) included the same cytokines as phase 1, except without SCF. During phase 3 (days 9-14), DOX was removed. For K562 cells, 50 μM of hemin was added and the cells were cultured for another 96 h. The CD71/CD235a kit (BD Biosciences) was used to detect erythroid lineage differentiation. All cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Wright-Giemsa staining. To assess the differentiation of different groups, HUDEP-2 cells were centrifuged for 300 x g at room temperature for 5 min, resuspended using 20 μl FBS (Stemcell Technologies, Inc.) and coated on the slide. After drying naturally at room temperature, Giemsa A and B solutions (Zhuhai Beso Biotechnology Co., Ltd.) were combined at a 1:1 volume ratio. The slide was washed with running water after staining for 5 min at room temperature, the cell morphology [including basophilic erythroblast (Baso-E), polychromatic erythroblast (Poly-E) and orthochromatic erythroblast (Ortho-E)] was observed and captured using Leica Aperiod CT6 microscope (Leica Microsystems GmbH).

Benzidine blue staining. The Hb expression level in K562 cells induced to differentiate by hemin was evaluated by mixing the cell suspension with a freshly made benzidine-H_2O_2 solution, consisting of 50 μl of 3% H_2O_2 (Sigma-Aldrich; Merck KGaA) and 0.4%/ml benzidine (Merck KGaA). After a 5-min treatment at room temperature, the cells were photographed under a light microscope (Olympus Corporation). The benzidine-positive cells were expressed as a percentage of at least 100 cells.

Fluorescence in situ hybridization (FISH). In HUDEP-2 cells, FISH was performed using specific probes for miR-6747-3p and BCL11A according to the manufacturer's instructions (Shanghai GenePharma Co., Ltd.). A total of ~5x10^4 HUDEP-2 cells were seeded on coverslip (NEST, Inc.; cat. no. 801007) in 24-well plates overnight. After which, the cells were washed with PBS and fixed in a 4% formaldehyde solution for 15 min at room temperature. The cells were incubated at room temperature with 0.1% buffer A for 10 min. After 15 min of incubation at 37˚C in Protein Free Rapid Blocking Buffer (EpiZyme, Inc.; cat. no. PS108), 1 μl of 1 μM FAM-labeled miR-6747-3p probe (5'-CTGGTGCAGAGGAAGGCAGGA-3') or 1 μM cy3-labeled BCL11A probe (5'-CCTGGTATTCTTAGCAGG TTAAAGG-3') with 73˚C rehydrated buffer E was added into the cells and incubated at 37˚C overnight in darkness. The next day, the cells were successively washed three times for 10 min: 0.1% buffer F at 37˚C, 2X buffer C at 60˚C, and 2X buffer C at 37˚C. 4',6'-DAPI was used to dye the cell nuclei for 10 min at room temperature. A Leica TCS SP8 CARS Confocal Microscope (Leica Microsystems GmbH) was used to identify the subcellular localization of miR-6747/BCL11A.

Luciferase reporter assay. The binding spot between miR-6747-3p and BCL11A was identified using the online tool Targetscan (version 8.0; https://www.targe tscan.org/vert_80/), miRWalk (version 3.0; http://mirwalk.umm.uni-heidelberg.de/) and miRDB (version V6; http://mirdb.org/miRDB/). Luciferase reporter vector pmiR-RB-REPORT™ (Promega Corporation) was inserted with wt-BCL11A and mut-BCL11A and co-transfected with HUDEP-2 cells with a density of 1x10^5 cells/well using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.). This includes the miR-6747-3p mimic, negative control, miR-6747-3p inhibitor and inhibitor negative control. The relative luciferase activity was calculated by the ratio of Renilla to firefly luciferase after incubation for 48 h at 37˚C. A total of three replicates were established for the experiment.

Western blotting. Proteins were extracted following the previously published method (32). Briefly, protein samples were obtained from HUDEP-2 and K562 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and quantified by BCA Kit (Beyotime Institute of Biotechnology). After being separated on a 12.5% SDS-PAGE gel, the protein samples (20 μg) were imprinted on a polyvinylidene difluoride membrane. Following 2 h of incubation at room temperature in Protein Free Rapid Blocking Buffer (EpiZyme, Inc.; cat. no. PS108), the membranes were then exposed to the following primary antibodies overnight at 4˚C: Anti-GAPDH (1:20,000; cat. no. ab8245; Abcam), anti-γ-globin (1:1,000; cat. no. ab156584; Abcam) and anti-BCL11A (1:1,000; cat. no. ab19487; Abcam). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5,000; Santa Cruz Biotechnology, Inc.; cat. no. SC-2005) and HRP-conjugated goat anti-rabbit IgG (1:5,000; Santa

Table I. Comparison of hematological parameters and biochemical indicators in patients with β-TM and healthy controls.

Characteristics	Control group	β-TM group	P-value
Sex (female/male)	65 (13/7)	60 (12/8)	0.744
Age, yrs	9.00±2.23	8.30±1.59	0.053
RBC, $x10^{12}$/l	4.65±0.24	3.71±0.42	<0.01
Hb, g/l	132.95±6.45	85.60±12.21	<0.01
MCV, fl	83.13±2.50	80.34±5.01	0.043
MCH, pg	28.62±0.91	26.33±2.07	<0.01
HbA, %	96.78±0.87	88.29±12.68	0.005
HbA2, %	2.79±0.04	3.49±0.50	0.169
HbF, %	0.15±0.08	8.22±2.86	0.007
PLT, $x10^{9}$/l	291.35±14.43	422.13±45.00	0.005

RBC, red blood cell; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; HbA, hemoglobin A; HbA2, hemoglobin A2; HbF, fetal hemoglobin; PLT, platelet.

Cruz Biotechnology, Inc.; cat. no. SC-2004) were utilized as the secondary antibodies at room temperature for 2 h. To identify proteins, the Chemiluminescence Western Blotting Detection system (Thermo Fisher Scientific, Inc.) was used. Densitometric analysis with Image J software (version 1.5; National Institutes of Health) was used to ascertain the relative expression of each protein.

Statistical analysis. GraphPad Prism 9.0 (Dotmatics) and SPSS 26.0 (IBM Corp.) were utilized for data analysis. The Kolmogorov-Smirnov test was employed to examine the normality of the data distributions. The differences between groups with normally distributed data (displayed as mean ± standard deviation) were assessed utilizing the two-tailed unpaired Student's t-test (2 groups) or the one-way ANOVA followed by Tukey's test (≥3 groups), as appropriate. The differences between groups without normally distributed data [displayed as the median and interquartile range M (P25, P75)] were tested by the Mann-Whitney U test (2 groups) or the Kruskal-Wallis test (≥3 groups), as appropriate. The association between hematological indicators and miR-6747-3p was examined using Spearman correlation analysis. Mean ± standard deviation was reported from three separate trials. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-6747-3p is upregulated in β-TM patients and associated with HbF. In the present study, 20 patients with β-TM and 20 healthy controls were recruited to confirm the expression of miR-6747-3p and evaluate its clinical significance. Compared with the healthy participants, patients with β-TM had significantly decreased Hb levels and higher HbF levels (Table I), which was consistent with the anemia phenotype of β-TM (33). There was a notable rise in the expression of MiR-6747-3p in patients with β-TM (Fig. 1A). Notably, correlations were evaluated between miR-6747-3p and hematological parameters (Fig. 1B), and HbA_2 was found to be associated with miR-6747-3p. However, miR-6747-3p was statistically not correlated with RBC, HGB, MCV, HbA and PLT. By dividing patients into HbF high (HbF, ≥2.7%) and low (HbF, <2.7%) expressing groups, it was discovered that patients with HbF ≥2.7% had significantly higher levels of miR-6747-3p than the patients with <2.7% HbF (Fig. 1C). Additional examinations uncovered that miR-6747-3p was significantly correlated with HbF levels in patients with β-TM (r=0.636; P<0.05; Fig. 1D). These results indicate that miR-6747-3p may play a role in the elevated HbF in patients with β-TM. Consequently, the effects of miR-6747-3p overexpression and knockdown in erythroid precursor cells were further investigated.

miR-6747-3p regulates cell proliferation and apoptosis of erythroid precursor cells. Electroporation transfection with overexpression and knockdown vectors for miR-6747-3p was performed on HUDEP-2 cells and K562 cells. A validation of cell transfection efficiency was provided in Fig. S1. The CCK-8 results showed that the absorbance of the miR-6747-3p mimic group was reduced in HUDEP-2 cells. Conversely, absorbance of the miR-6747-3p inhibitor group exceeded the NC inhibitor group (Fig. 2A). Similar results were detected in K562 cells (Fig. 2B), inferring that miR-6747-3p expression decreases cell proliferation in HUDEP-2 cells and K562 cells.

Cell cycle analysis showed that miR-6747-3p mimic cells led to higher percentages of the S phase, while the inhibitor group had the opposite effect (Fig. 2C and D). Consistent with the cell growth findings where miR-6747-3p demonstrated the capability to reduce cell growth in a laboratory setting, these findings suggested that overexpression of miR-6747-3p results in a halt from S to G2/M phase in the cell cycle.

Apoptosis was examined in HUDEP-2 and K562 cells with either overexpression or knockdown of miR-6747-3p to explore its role in cell apoptosis. The results indicated that the apoptosis rate was increased in the miR-6747-3p mimic group in both HUDEP-2 and K562 cells (P<0.5). By contrast, the group treated with the miR-6747-3p inhibitor had a lower rate of apoptosis than the group treated with the NC inhibitor

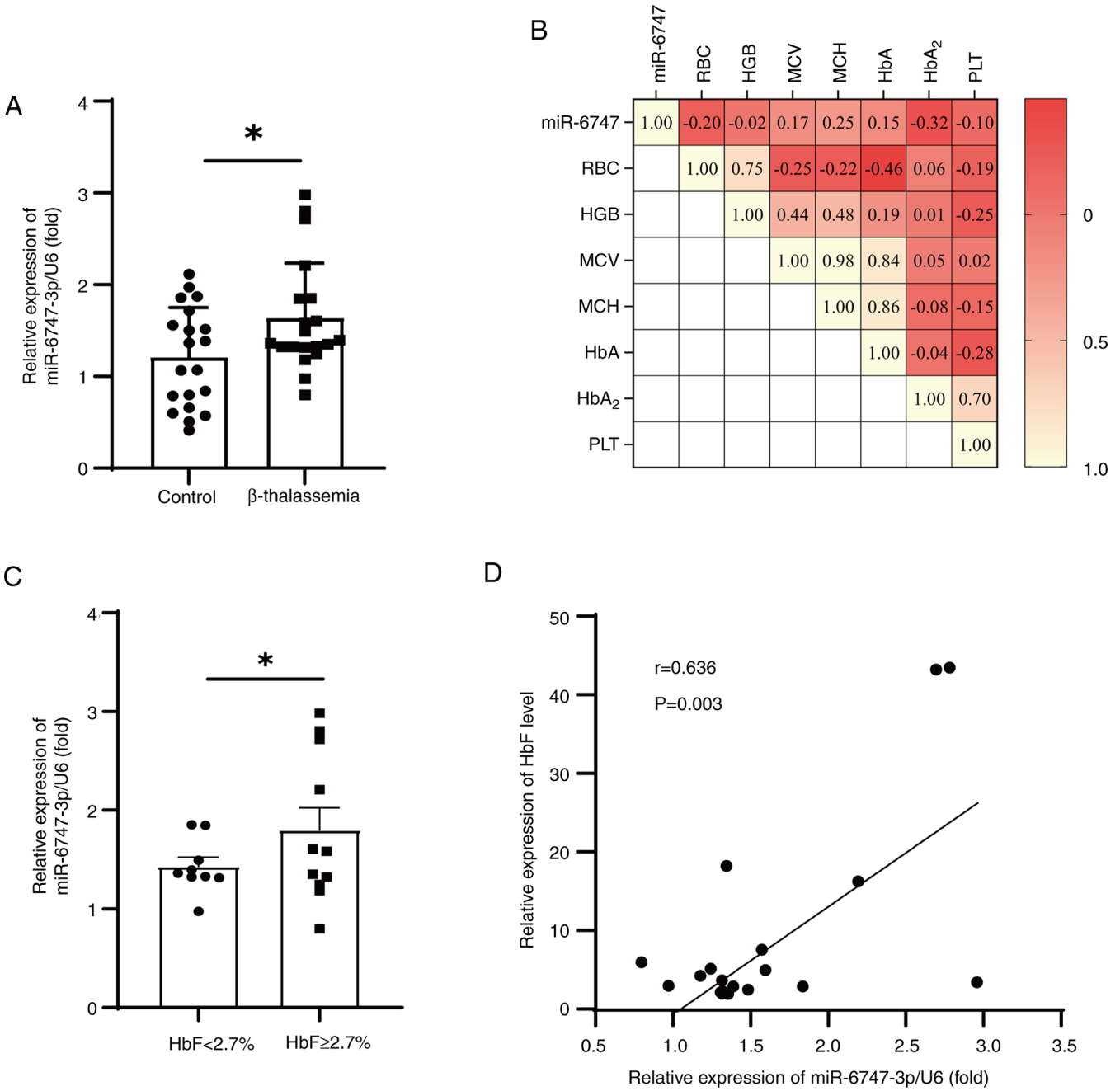

Figure 1. Examining the miR-6747-3p expression level and its association with various clinical markers. (A) Comparative analysis of miR-6747-3p expression between patients with β-TM (n=20) and healthy individuals (n=20). (B) Correlation analysis of miR-6747-3p with clinical indicators in patients with β-TM. (C) Examination of miR-6747-3p expression across different HbF levels in patients with β-TM (n=10). (D) Correlation analysis showed a significant positive correlation between miR-6747-3p expression level and HbF level *P<0.05. RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; HbA, hemoglobin A; HbA2, hemoglobin A2; HbF, fetal hemoglobin; PLT, platelet; HGB, hemoglobin.

(P<0.5; Fig. 2E and F). The aforementioned results indicate that miR-6747-3p induces cell cycle arrest and apoptosis, thereby reducing cell proliferation.

miR-6747-3p controls the maturation of precursor cells. The transferrin receptor CD71 is abundantly present in early erythroid cells, whereas the surface marker CD235a becomes more prominent as erythroblasts mature (34). After 14 days of terminal differentiation, flow cytometry analysis revealed that 71.3±2.77% of the differentiated erythroid precursors in the miR-6747-3p mimic group expressed CD71/CD235a, a notably higher percentage compared with the NC mimic group (65.3±2.20%; Fig. 3A), while the cell differentiation rate was reduced in HUDEP-2 cells treated with the miR-6747-3p inhibitor compared with the NC inhibitor group (69.5±3.35% vs. 81.2±2.66%; Fig. 3B).

Wright-Giemsa was applied to stain cultivated erythroblasts. Morphological analysis of HUDEP-2 cells using miR-6747-3p mimics revealed a concomitant increase in Orth-E (Fig. 3C), whereas the miR-6747-3p inhibitor group failed to progress beyond the Poly-E stage of differentiation at day 14 (Fig. 3D).

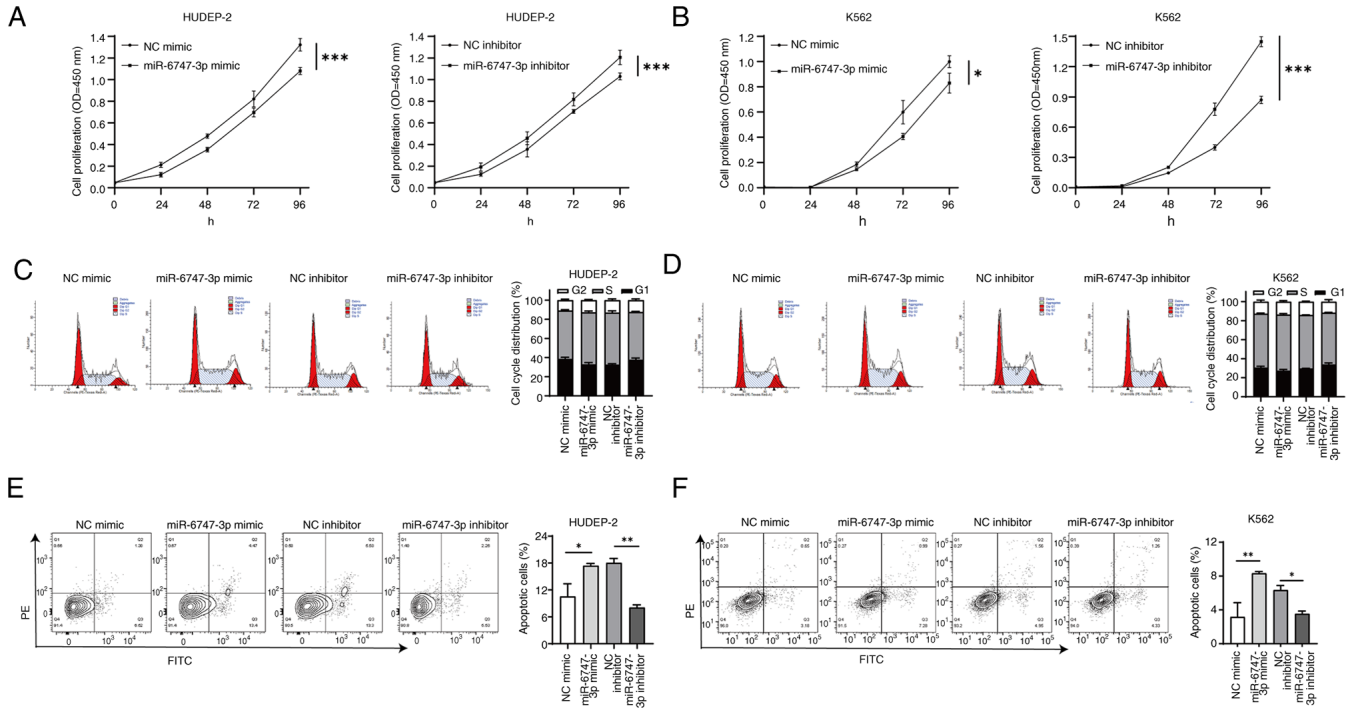

Figure 2. Impact of miR-6747-3p on the proliferation, cell cycle and apoptosis of HUDEP-2 cells and K562 cells. (A) Effect of overexpression and knockdown of miR-6747-3p on HUDEP-2 cell proliferation. (B) Effect of overexpression and knockdown of miR-6747-3p on K562 cell proliferation. (C) Examining the impact of miR-6747-3p upregulation and downregulation on the cell cycle of HUDEP-2 cells. (D) Examining the impact of upregulating and downregulating miR-6747-3p on the cell cycle of K562 cells. (E) Examining the impact of miR-6747-3p upregulation and downregulation on apoptosis in HUDEP-2 cells. (F) Impact of miR-6747-3p upregulation and downregulation on apoptosis in K562 cells (three independent experiments). *P<0.05; **P<0.01; ***P<0.001. NC, negative control; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

After 96 h of co-culture with hemin, the miR-6747-3p mimic group showed a greater percentage of positive K562 cells in the Benzidine blue staining compared with the NC group. In Fig. 3E, the miR-6747-3p inhibitor group showed a lower positive rate than the NC inhibitor group. Flow cytometry results showed a rise in CD71/CD235a^{+} cells in the miR-6747-3p mimic group compared with the NC group while the differentiation rate of the miR-6747-3p inhibitor group was lower than that of NC inhibitor group (Fig. 3F), indicating that miR-6747-3p speeds up erythroid differentiation.

miR-6747-3p induces HbF expression of erythroid precursor cells. It was previously confirmed that miR-6747-3p can enhance the development of HUDEP-2 and K562 cells, and is associated with the levels of HbF in patients with β-TM. Next, F-cell detection in HUDEP-2 cells was performed to further confirm whether miR-6747-3p could regulate the expression of HbF. The expression of HbF was subsequently quantified after 14 days of differentiation in each group. The results indicated that miR-6747-3p could strongly induce γ-globin in erythroid precursor cells. The miR-6747-3p mimic group (55.1±0.76%) had significantly higher HbF expression than the NC group (47.1±0.62%; P<0.05), while the miR-6747-3p inhibitor group had significantly lower HbF expression (44.0±0.47% vs. 37.2±1.80%; P<0.05; Fig. S2).

BCL11A is the direct target of miR-6747-3p. TargetScan, miRwalk and miRDB were employed to forecast the mRNA targets of miR-6747-3p to illustrate the molecular mechanism of generating HbF expression. According to the results, the anticipated target mRNA numbers were 820, 6,247 and 4,392, according to the sequence. Among the three programs, a total of 326 mRNA targets were shared (Fig. 4A). The erythroid-related transcription factor BCL11A was selected as a possible target gene for previous publications showing the negative regulation of HbF (35-39). RT-qPCR was used to measure BCL11A mRNA levels. The results showed that, in comparison to normal controls, patients with β-TM had considerably lower levels of BCL11A mRNA (Fig. 4B). Furthermore, BCL11A mRNA was found to have a negative correlation with both γ-globin (r=-0.637; P<0.05; Fig. 4C) and miR-6747-3p (r=-0.567; P<0.05; Fig. 4D). In addition, the colocalization of miR-6747-3p and BCL11A in HUDEP-2 cells was confirmed by fluorescence in situ hybridization assay (Fig. 4E), suggesting that the target gene of miR-6747-3p was BCL11A.

Whether miR-6747-3p directly interacts with BCL11A was also examined. The CCGUCC binding site in miR-6747-3p targeting GCAGGA in BCL11A 3'-UTR was identified using Targetscan (Fig. 4F). Thus, HUDEP-2 cells were co-transfected with miR-6747-3p mimic and a pmiR-RB-REPORT™ plasmid with the wild-type BCL11A 3'-UTR. The miR-6747-3p mimic significantly decreased the luciferase activity, according to the results. This interaction was further validated by demonstrating that it was eliminated when the BCL11A 3'-UTR binding region was mutated from GCAGGA to CCGTCC (Fig. 4G). By contrast, the luciferase activity of the BCL11A seed region was notably higher in the miR-6747-3p inhibitor group, with no significant change observed in the mutant group. Briefly, miR-6747-3p was able to directly attach to the 546-552 loci of the BCL11A 3'-UTR.

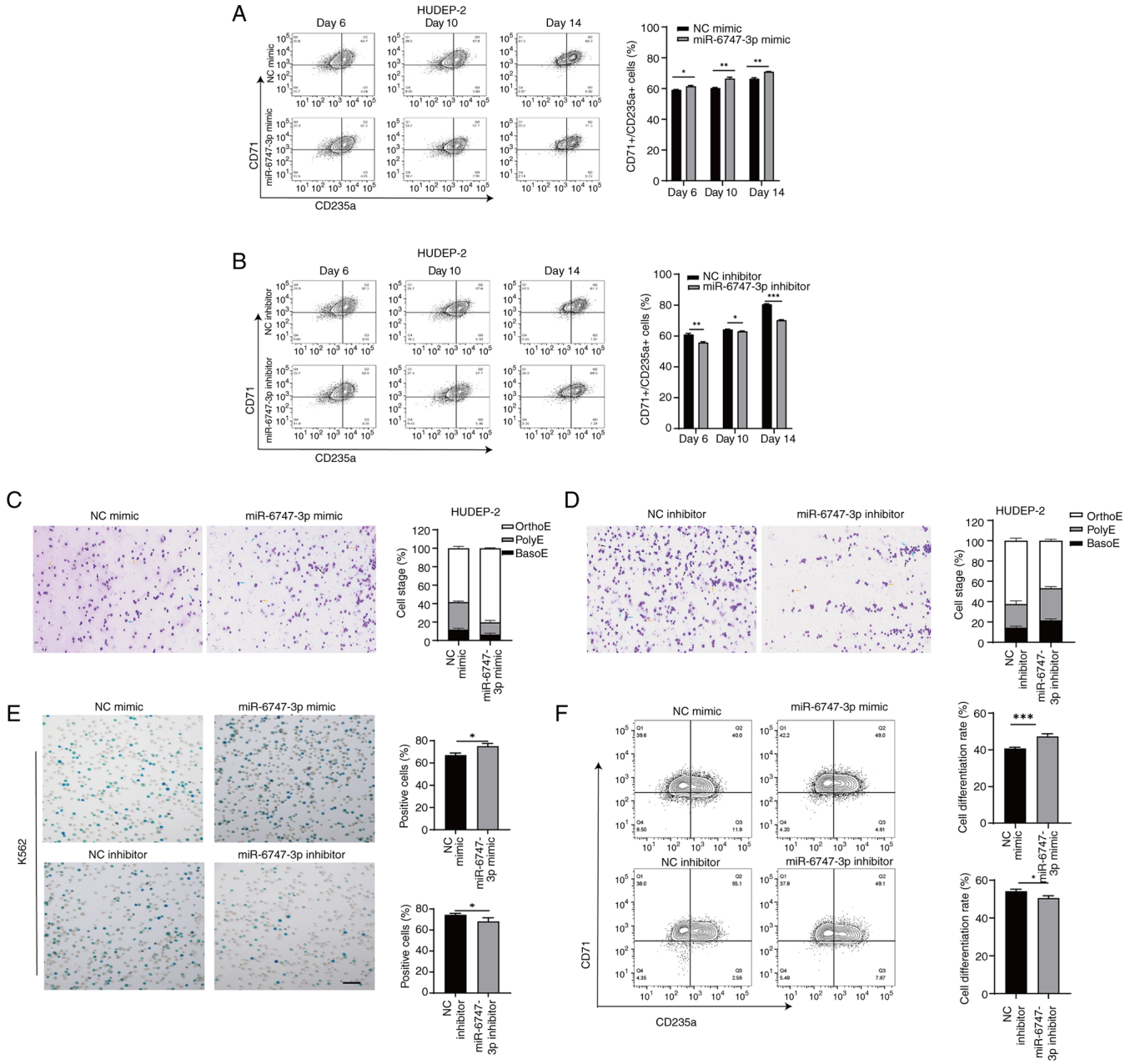

Figure 3. Impact of miR-6747-3p on the process of erythroid differentiation in both HUDEP-2 and K562 cell lines. (A) Impact of increased miR-6747-3p levels on the process of red blood cell maturation in HUDEP-2 cell line. (B) Impact of reducing miR-6747-3p on the process of erythroid differentiation in HUDEP-2 cells. (C) Representative images of HUDEP-2 cells transfected with miR-6747-3p and NC mimic stained with Wright-Giemsa 14 days post-erythroid differentiation. (D) Representative images of HUDEP-2 cells transfected with miR-6747-3p and NC inhibitor stained with Wright-Giemsa 14 days post-erythroid differentiation (blue, basophilic normoblasts; yellow, polychromatic normoblasts; black, orthochromatic normoblasts; magnification, x40). (E) The cell differentiation in transfected K562 cells was presented via benzidine staining (magnification, x20). (F) Impact of miR-6747-3p upregulation and downregulation on the process of erythroid differentiation in K562 cells (three independent experiments). *P<0.05; **P<0.01; ***P<0.001. NC, negative control.

miR-6747-3p targets BCL11A to increase the expression of γ-globin. Fig. 5 illustrates how the transfection of miR-6747-3p mimics into HUDEP-2 (Fig. 5A) and K562 (Fig. 5B) cells reduces BCL11A transcripts and increases γ-globin mRNA levels. Notably, a >2-fold elevation of BCL11A mRNA in HUDEP-2 (Fig. 5C) and K562 (Fig. 5D) cells in the miR-6747-3p inhibitor group was observed, while γ-globin mRNA expression was significantly decreased. These results were also corroborated at the protein level. Western blot analysis showed a notable increase in BCL11A protein levels in the miR-6747-3p inhibitor group compared with the NC inhibitor group, while γ-globin levels were significantly decreased (Fig. 5E and F). These findings indicate that miR-6747-3p can inhibit the expression of BCL11A in both HUDEP-2 and K562 cells.

Discussion

miRNAs are essential for controlling the expression of Hb as well as several biological processes, such as erythropoiesis and cell proliferation (40). In the present study it was first demonstrated that β-TM had a markedly elevated expression level

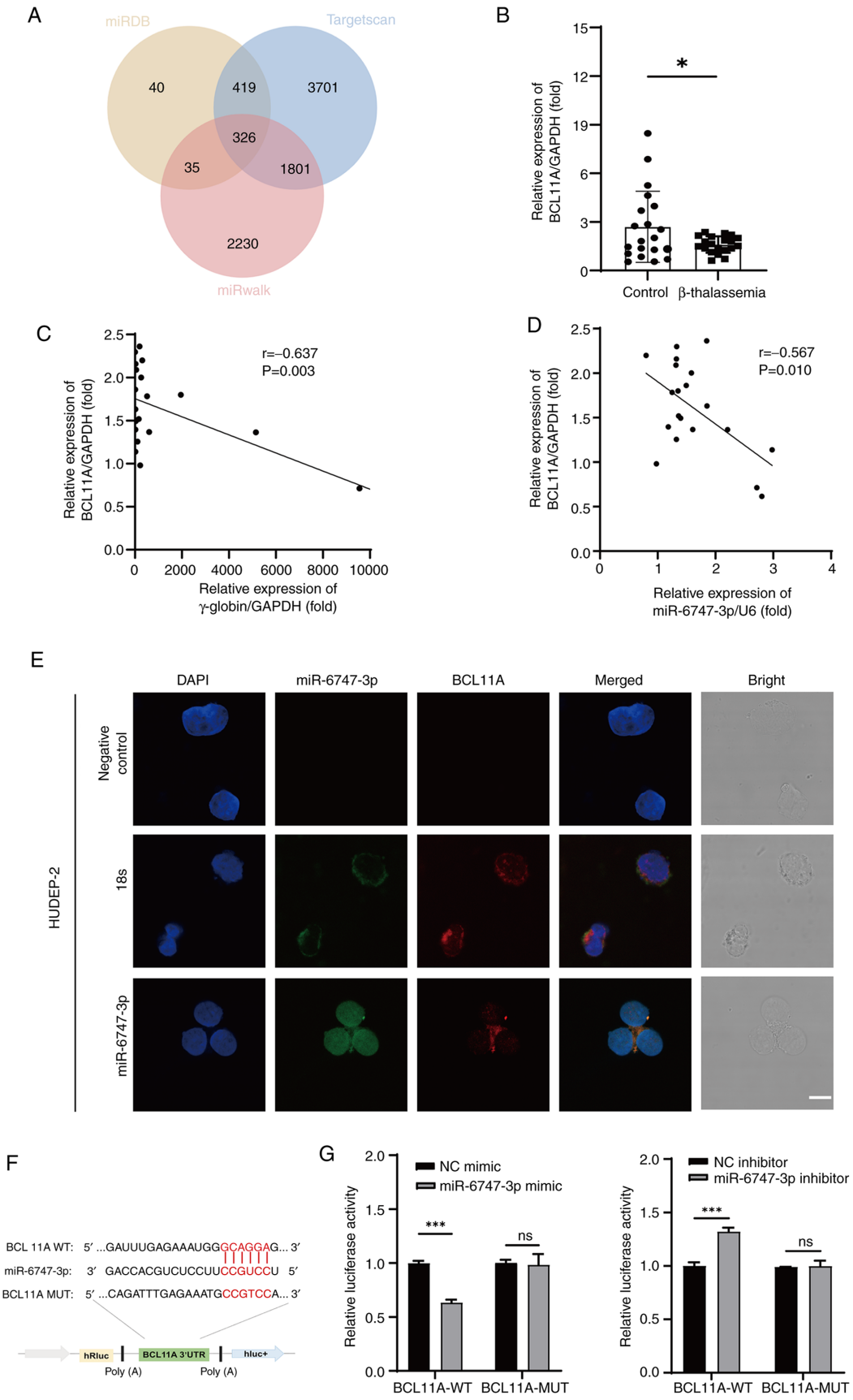

Figure 4. miR-6747-3p targets the BCL11A gene. (A) Prediction of miRNA 6747-3p targets using miRDB, TargetScan and miRwalk. (B) Low expression of BCL11A mRNA in patients with β-TM (n=20). (C) Correlation analysis showed a significant negative association between γ-globin and BCL11A (n=20). (D) Correlation analysis showed a significant negative association between miR-6747-3p and BCL11A (n=20). (E) Colocalization of miR-6747-3p and BCL11A in HUDEP-2 cells detected by FISH assay. FAM-labeled the miR-6747-3p probes (green), Cy3 labeled the BCL11A probe (red) and DAPI stained the nuclei (blue). (magnification, x1,000; scale bar, 10 μm) (F) The binding sites identified in BCL11A mRNA are complementary to miR-6747-3p. (G) The dual luciferase gene reporter assay results show that miR-6747-3p can attach to the 3'-UTR of BCL11A mRNA (three independent experiments). *P<0.05; ***P<0.001. HbF, fetal hemoglobin; WT, wild type; MUT, mutant; ns, not significant.

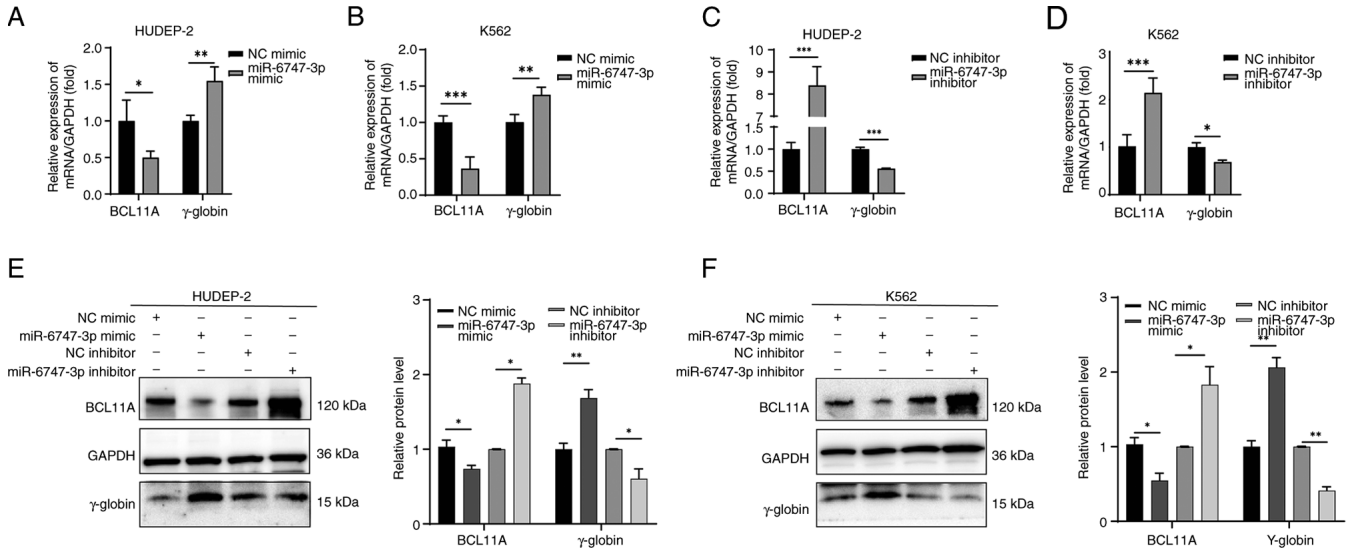

Figure 5. Effects of miR-6747-3p on the expression of BCL11A and γ-globin. (A) Analysis of BCL11A and γ-globin mRNA expression in HUDEP-2 cells transfected with the miR-6747-3p mimic. (B) BCL11A and γ-globin mRNA expression analysis of K562 cells in the miR-6747-3p mimic group. (C) BCL11A and γ-globin mRNA expression analysis on HUDEP-2 cells in the miR-6747-3p inhibitor group. (D) BCL11A and γ-globin mRNA expression investigation into K562 cells in the miR-6747-3p inhibitor group. (E) Expression analysis of BCL11A and γ-globin protein in HUDEP-2 cells. (F) Expression analysis of BCL11A and γ-globin protein in K562 cells (three independent experiments). *P<0.05; **P<0.01; ***P<0.001. NC, negative control; BCL11A, B-cell lymphoma/leukemia 11A.

of miR-6747-3p. Based on correlation analysis, miR-6747-3p has a significant positive correlation with HbA_2 and HbF. Overexpression of hsa-miR-6747-3p impedes cell growth by causing cell cycle arrest, inducing cell apoptosis, accelerating erythroid differentiation and increasing HbF expression. In addition, it was shown that miR-6747-3p negatively controls BCL11A by binding to the 546-552 loci of BCL11A mRNA 3'-UTR. The aforementioned results indicated that miR-6747-3p may be an essential regulator of the HbF level via modulating BCL11A expression.

Numerous researchers have explored the interaction between miRNA and HbF reactivation in β-thalassemia. For example, *let-7/LIN28*, miR-138 and miR-210 elevate γ-globin expression, whereas miR-223-3p, miR-150 and miR-146a suppress γ-globin production (41-43). Single nucleotide polymorphisms in miRNA target genes may also lead to abnormal Hb expression (44). These findings imply that miRNAs may be valuable biomarkers for β-TM diagnosis and prognosis. miR-6747-3p expression was studied in several diseases. In patients with endometriosis, miR-6747-3p showed a good diagnostic capability for infertility combined with ultrasonography (26). Another cohort study demonstrated a noteworthy correlation between miR-6747-3p and SCLC by targeting the colony-stimulating factor 3 receptor, a crucial component of cellular autophagy factor, which was highly associated with myeloid and lymphoid leukemias (45). In a previous study, the research team identified upregulated miR-6747-3p expression in patients with β-TM by microRNA sequencing (25). However, whether miR-6747-3p plays a role in β-thalassemia remains unknown. The present analysis of miR-6747-3p expression levels revealed higher levels in patients with β-TM (average age, 8.30±1.59 years) compared with healthy controls (average age, 9.00±2.23 years), aligning with previous findings (25). Moreover, miR-6747-3p has a significant positive correlation with HbF.

The effect of miR-6747-3p overexpression/knockdown on cell cycle, apoptosis, differentiation and proliferation were examined by CCK8, flow cytometry, Wright-Giemsa and benzidine staining tests. The results revealed that miR-6747-3p overexpression inhibited cell growth, accelerated apoptosis and stimulated cellular erythroid differentiation. Ineffective erythropoiesis, a prevalent condition in β-thalassemia, is characterized by high cell proliferation (46). Prior research on miRNA variance examination in thalassemia indicated that the level of miRNA-101-3p was notably elevated in $CD34^{+}$ cells separated from peripheral blood of patients with thalassemia, with a more pronounced impact observed in individuals with thalassemia minor compared with major patients and healthy controls (47). However, in the present results, the increased expression of miRNA-6747-3p inhibited cell proliferation, which was speculated to be related to the cell variance, and the present experimental group were all made up of patients with thalassemia major. Moreover, it is noteworthy that the change in 450 nm absorbance was more pronounced in K562 cells than in HUDEP-2 cells. The miR-6747-3p mimic group in K562 cells exhibited a notably increased apoptosis rate compared with the other three groups. The variable expression patterns of miRNAs and their capacity to modify physiological processes within cells may be a contributing factor to this phenomenon. Subsequent experiments with superior red lineage cells ($CD34^{+}$) and relevant subgroups of patients with minor and intermediate thalassemia are required.

By contrast, inhibition of miR-6747-3p was also shown to have an impact on erythroid precursor cells, perhaps reducing cell cycle arrest. This finding needs to be validated by other experiments involving cell cycle-related proteins. Furthermore, examining the morphological changes during erythroid differentiation showed that miR-6747-3p inhibitor cells had a greater number of basophilic erythroblasts and a lower number of orthochromatic erythroblasts compared with the control groups

at the end of 14 days in HUDEP-2 cells. The multistep process of erythropoiesis involves committing multipotent HSCs to develop into the red blood cell lineage (34). It is reasonable to infer that miR-6747-3p regulates the differentiation of erythroid precursor cells and ameliorates symptoms of anemia. K562 cells, first discovered in a patient with chronic myeloid leukemia, are frequently utilized as a laboratory model for studying the molecular processes in human globin gene expression and assessing the effectiveness of novel medications that promote differentiation (48,49). As first demonstrated by Rutherford *et al* (50), K562 cells have a low potential for Hb-synthesizing but can undergo erythrocyte differentiation in response to various compounds, such as hemin. In the present study, benzidine blue staining results demonstrate that miR-6747-3p overexpression in K562 cells leads to an apparent increase proportion of benzidine-positive cells, whereas inhibiting miR-6747-3p leads to the opposite effect, suggesting that miR-6747-3p promotes Hb synthesis in K562 cells.

In previous decades, efforts to increase HbF synthesis have been motivated by the concept that higher HbF diminishes the severity of β-thalassemia (38,40). Liu *et al* (38) found that overexpression of miR-486-3p could notably decrease BCL11A protein and enhance the synthesis of γ-globin. Likewise, miR-210 boosts the synthesis of γ-globin by reducing the levels of BCL11A in erythroid progenitors derived from patients with β-thalassemia (40). Furthermore, in K562 cells, miR-210 can enhance the suppression of BCL11A induced by mithramycin (51). The BCL11A gene is mainly found in the brain and hematological organs, and it is situated on chromosome 2p16.1 (52,53). Research has indicated that BCL11A is essential for regulating the transition of Hb and preserving the inactivity of the γ-globin gene (54,55). An attempt has been made to reactivate HbF through BCL11A knockdown by synthesizing BCL11A short hairpin RNA that inserts into the flanking region of the miRNA precursor. *In vitro* research combined with *in vivo* mouse models have validated the shRNA-based treatment (56). Based on the aforementioned research, β-thalassemia may be treated using miRNA-based targeted therapies (57). In the present study, patients diagnosed with β-TM exhibited decreased levels of BCL11A mRNA expression. Moreover, the Pearson correlation test found that BCL11A levels were negatively correlated with miR-6747-3p and HbF. Therefore, it was hypothesized that hsa-miR-6747-3p may be involved in regulating HbF expression by targeting BCL11A.

Through the application of bioinformatics methods, it was discovered that miR-6747-3p could bind to the 546-552 positions on the 3'-UTR of BCL11A mRNA. This finding was validated through fluorescence in situ hybridization. In HUDEP-2 cells, miR-6747-3p and BCL11A were discovered extensively distributed in the cytoplasm. Furthermore, the luciferase results indicated that miR-6747-3p could directly interact with the 546-552 region of the BCL11A mRNA 3'-UTR. Of note, miR-6747-3p was found to decrease BCL11A levels and increase γ-globin expression in HUDEP-2 and K562 cells, as shown by RT-qPCR and Western blot analyses. These results confirm that miR-6747-3p in β-TM could target BCL11A directly.

Overall, the results of the present study indicated that miR-6747-3p has a specific clinical utility for β-TM. The statistical analysis revealed a notable molecular pathway that includes miR-6747-3p, BCL11A and γ-globin. Despite the small sample size recruited in the present study, this newly identified translational regulatory mechanism may offer a significant target for synthesizing HbF by mimicking miR-6747-3p functions. Notably, HbF levels can be influenced by drugs, autoimmune disease, pregnancy, malignancy, diabetes, genetic modifiers and hematological disorders and splenic dysfunction (44). Several SNPs in BCL11A, such as rs4671393, rs4127407 and rs7606173, are associated with decreased HbF levels but can also lead to elevated HbF levels via microdeletion (37). Previous research found no significant variations in hematological parameters between rs1426407, rs1018987 and rs11886868 (58). However, 3.3% of HbF level variation in β^0-thalassemia/HbE among Thai patients was found to be strongly correlated with rs6729815 (59). Additionally, SNPs rs6545816 (A/C), rs6545817 (A/G), rs766432 (A/C) and rs6729815 (A/G) were linked with high HbF levels (59). Given that mutations at the BCL11A locus can influence HbF expression, future research should implement more rigorous inclusion criteria. In the future, the miR-6747-3p-BCL11A-γ-globin axis in β-thalassemia will be investigated in more detail by increasing the sample size and conducting *in vivo* studies.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

AL, MC, SZ and WZ performed all the experiments and collected the data. JL was responsible for analyzing the

data. SL and YZ assisted in the cell culture. NL, LX and HH conceived and designed the study. AL and SZ wrote the main manuscript. LX and HH supervised the study and confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was conducted following the Declaration of Helsinki, and approved by The Ethics Committee of The Fujian Maternity and Child Health Hospital (Fuzhou, China; approval no. 2019073). All patients or their guardians provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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